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Antihyperlipidemic effect of Fu-Ling-Pi is associated with abnormal fatty acid metabolism by UPLC-HDMS-based lipidomics

Hua Miao[a,b], Ming-Hua Li[d], Xu Zhang[a], Sheng-Jun Yuan[a], Charlene C. Ho[c], Ying-Yong Zhao[a,b,*]

[a] Key Laboratory of Resource Biology and Biotechnology in Western China, Ministry of Education, Northwest University, No. 229 Taibai North Road, Xi'an, Shaanxi 710069, China

[b] Division of Nephrology and Hypertension, School of Medicine, University of California, Irvine, Med Sci I, C352, UCI Campus, Irvine, CA, 92897, USA

[c] Department of Biochemistry, Li Ka Shing Faculty of Medicine, The University of Hong Kong, 21 Sassoon Road, Pokfulam, Hong Kong, China

[d] National Institutes for Food and Drug Control, State Food and Drug Administration, No. 2 Tiantan Xili, Beijing, 100050, China

Corresponding author: Ying-Yong Zhao, PhD, MD, Professor, Tel: +86 29 88304569; Fax: +86 29 88304368; E-mail: zyy@nwu.edu.cn; zhaoyybr@163.com (Y.Y. Zhao)

Abstract

The surface layer of *Poria cocos* (SLPC), a traditional Chinese medicine, has been commonly used for diuretic and antihyperlipidemia in Asia. In order to understand its biochemical mechanism of antihyperlipidemia, a lipidomic approach based on ultra-performance liquid chromatography coupled with a quadrupole time-of-flight synapt high-definition mass spectrometry was carried out to characterize the plasma lipid metabolic profile of antihyperlipidemic effect of SLPC in rats fed with high fat diet. Lipid metabolites with significant changes were characterized as potential biomarkers associated with the development of hyperlipidemia and antihyperlipidemia of SLPC using partial least-squares-discriminate analysis, heatmap display, correlation coefficient analysis and receiver-operating characteristic curves. The analysis of the biological pathway was performed with metabolomics pathway analysis (MetPA). The lipid metabolic profile of hyperlipidemia rats separated from control rats and SLPC treated rats were closer to the control rats. Six lipid metabolites including five fatty acyl lipids palmitic acid, dodecanoic acid, L-palmitoylcarnitine, oleoylcarnitine and linoleyl carnitine and one sphingolipid phytosphingosine were considered as biomarkers of diet-induced hyperlipidemia and antihyperlipidemic effect of SLPC. MetPA revealed that identified lipid biomarkers were responsible for diet-induced hyperlipidemia and antihyperlipidemic effect of SLPC. These biomarkers were associated with fatty acid metabolism, fatty acid biosynthesis, sphingolipid metabolism, fatty acid elongation in mitochondria and unsaturated fatty acids biosynthesis. The findings suggest that a high fat diet led to the perturbation of fatty acid metabolism and sphingolipid metabolism, which may be the pharmacological basis of an antihyperlipidemic effect of SLPC.

Keywords: *Poria cocos* epidermis; hyperlipidemia; lipidomics; ultra-performance liquid chromatography; high-definition mass spectrometry; fatty acid metabolism

Introduction

Hyperlipidemia is caused mainly due to the increase in serum total cholesterol (TC), triglyceride (TG) and low-density lipoprotein cholesterol (LDL-C) as well as decrease in high-density lipoprotein cholesterol (HDL-C). It is a critical risk factor for cardiovascular and cerebrovascular diseases. It is closely associated with hypertension, obesity and coronary heart disease. Hyperlipidemia can be treated with medications and dietary alterations that alter abnormal complex lipid metabolic pathways. Statins (hydroxy methylglutaryl coenzyme A reductase

inhibitors), fibrates (phenoxy aromatic acids) and nicotinic acid are usually regarded as lipid-lowering drugs used in prevention and treatment of hyperlipidemia. However, these drugs have certain side effects such as muscle and liver damage.

Traditional Chinese medicine (TCM) may play an important role in the prevention and treatment of various diseases. More than 2000 plants are used in traditional medicine or alternative medicine and some plants are used in treatment of cardiovascular diseases including hyperlipidemia and ischemic heart disease [1]. TCM is a medical system with the main feature of the therapeutic effects of multi-component drugs that can hit multiple targets with multiple chemical components. It produces a holistic therapeutic effect via a multi-ingredient prevention or treatment to enhance therapeutic efficacy and reduce toxicity or side effects. Despite the great progress in the search of bioactive fractions and compounds from TCM, there is still a bottleneck in the development of novel methods to illuminate the integral therapeutic efficacy and synergism of a multi-component TCM regime.

Lipidomics, as a part of systems biology and a branch of metabolomics, is an analytical approach to holistic investigation of a multi-parametric response of living systems based on the lipid metabolic profiling in the complex biological samples [2]. Lipidomics provides the variation of whole lipid metabolic networks for observing pathological states, providing diagnostic information, demonstrating drug therapeutic efficacy and illuminating biochemical mechanism in animals and human [3,4,5,6]. Mass spectrometry and chromatography techniques have greatly promoted the developments and applications of lipidomics [7,8]. Among those analytical techniques, ultra-performance liquid chromatography coupled with quadrupole time-of-flight synapt high-definition mass spectrometry (UPLC-QTOF/HDMS) is most suitable for lipidomics, especially for untargeted lipid profiles due to its enhanced reproducibility of retention time [9,10,11,12]. Lipidomics have shown great potential in the application of therapeutic effects of the holistic approach in TCM treatment [13,14,15].

Poria cocos is a well-known TCM that has frequently been prescribed as one of the main ingredients in TCM's compound prescriptions. About ten percent of the TCM prescriptions admitted to Chinese Pharmacopoeia contain *poria cocos*. As reported previously, the chemical components of *Poria cocos* include triterpenes, polysaccharides and steroids [16,17]. However, the triterpenoid is the main component of the surface layer of *Poria cocos* (SLPC) of the sclerotia. It was used for promoting urination and to leaving out dampness, thus alleviating the problems caused by stagnation from dampness such as edema and urinary dysfunction [18,19,20]. Our recent study demonstrated the ethanol extracts of SLPC had a remarkable diuretic and nephroprotective

effect [21,22]. To best of our knowledge, no published report investigated antihyperlipidemic effect of SLPC and its action mechanism. In the present study, the hyperlipidemia model was induced in rats with a high fat diet. A sensitive UPLC-QTOF/HDMS was used to investigate the antihyperlipidemic effect of SLPC on the diet-induced hyperlipidemic rats. Partial least squares discriminant analysis (PLS-DA), principal component analysis (PCA), correlation analysis, heatmap display, receiver-operating characteristic (ROC) curves validation and metabolomics pathway analysis (MetPA) were utilized to investigating the antihyperlipidemic effect of SLPC and to clarify the biochemical mechanism of antihyperlipidemic effect of SLPC. To date, this is the first report of antihyperlipidemic effect of SLPC from diet-induced hyperlipidemic rats using a metabolomic approach. Five fatty acyl lipids including palmitic acid, dodecanoic acid, L-palmitoylcarnitine, oleoylcarnitine and linoleyl carnitine and one sphingolipid phytosphingosine were considered as biomarkers of diet-induced hyperlipidemia and antihyperlipidemic effect of SLPC. These findings suggest a high fat diet led to the perturbation of fatty acid metabolism, which may be the pharmacological basis of antihyperlipidemic effect of SLPC.

Materials and methods

Animals and sample collection

Male SD rats (Fourth Military Medical University, Xi'an, China) were randomly assigned into a control (CTL), hyperlipidemia (HLE) or hyperlipidemia with SLPC treatment groups (HLE+SLPC) (n=8/group). The CTL group was maintained on regular diet throughout the experimental period. The HLE group was fed high fat diets for six weeks [23]. The diet-induced HLE group and HLE+SLPC group was fed a high fat diets containing 7.5% lards, 10% egg yolk powder, 0.3% sodium cholate, 1% cholesterol and 0.2% methylthiouracil for six weeks. The HLE+SLPC group was administered SLPC (60 mg/ml) by gastric irrigation for six weeks. After six weeks, blood samples were obtained by carotid artery cannulation and stored at −80 °C.

Serum biochemistry

Serum TC, TG, HDL-C and LDL-C concentrations were measured with an Olympus AU640 automatic analyser based on Olympus AU640 user manual.

Sample preparation

Lipid extractions were performed in an Ostro 96-well plate using a single-step in-well extraction as previously described [24]. Briefly, 0.1 ml of plasma was loaded into each well of a two ml Ostro sample preparation plate fitted onto a vacuum manifold. 0.3 ml of elution solvent (1:1, $CH_3OH/CHCl_3$) was added to each well and mixed

by aspirating the mixture 10x using a micropipette. A vacuum of 15'' Hg was applied to the plate until the solvent was drained completely. This step was repeated with another 0.3 ml of $CH_3OH/CHCl_3$ with the fraction 3 times. The eluate was dried by nitrogen, redissolved with $CH_3OH/CHCl_3$ for UPLC-MS analysis.

UPLC-MS analysis

The UPLC-MS analysis was performed on a Waters Acquity^{TM} Ultra Performance LC system equipped with a Waters Xevo^{TM} G2 QTof MS. UPLC separation was carried out on a HSS T3 (2.1 mm×100 mm, 1.8 μm) column. The mobile phase consisted of 10 mmol/L ammonium formate and 0.1% formic acid in 2-propanol/acetonitrile (90/10) (A) and 10 mmol/L ammonium formate and 0.1% formic acid in acetonitrile/water (60/40) (B) was used as follows: a linear gradient from 0–10 min, 40.0–99.0% A and 10.0–12.0 min, 99.0–40.0% A. The flow rate was 0.5 ml/min. The autosampler and column temperatures were set at 4 °C and 55 °C, respectively. A 2.0 μl sample solution was injected for each run. The scan ranged from 50 to 1200 *m/z* in positive ion mode. The cone and capillary voltage were set at 45 V and 2.5 kV, respectively. The desolvation gas was set at 900 L/h at a temperature of 550 °C; the cone gas was set at 50 L/h and the source temperature was set at 120 °C. All the acquisitions were operated by Waters MassLynx v4.1 software.

Pattern recognition analysis and data processing

To assess the developed method, the precision and reproducibility were performed as described in detail previously [25]. The acquired data were imported to Markerlynx XS for peak detection and alignment. The data were normalized to the summed total ion intensity per chromatogram, and the resultant data matrices were introduced to the EZinfo 2.0 for PLS-DA. Lipid metabolites were extracted from PLS-DA, and the potential biomarkers were chosen based on their contribution to the variation. Correlation analysis, heatmap display AND fold changes (HLE group/CTL group, HLE+SLPC group/HLE group or HLE+SLPC group/CTL group) and the area under the curve (AUC) from the identified lipid metabolites were produced or calculated by Metaboanalyst 3.0. Ingenuity pathway analysis (IPA) was performed with metabolomics pathway analysis (MetPA) for pathway analysis and visualization metabolomics. One-way analysis of variance (ANOVA) and Mann-Whitney U-test were used to calculate the statistical significance by SPSS 19.0. False discovery rate (FDR) correction was calculated to reduce the risk of a false-positive by the adjusted p values (<0.05) based on the Benjamini Hochberg method.

Results and discussion

Clinical chemistry

The concentrations of TC, TG and LDL-C of the CTL rats were 2.59, 0.61 and 1.69 mmol/L, respectively. Compared to the CTL rats, the concentrations of TC (4.57 mmol/L), TG (0.97 mmol/L) and LDL-C (3.08 mmol/L) were significantly greater in the HLE rats. Similarly, the concentration of HDL-C of the CTL rats was 0.95 mmol/L whereas the concentration of HDL-C (0.57 mmol/L) was significantly decreased in the HLE rats. The serum TC, TG, LDL-C and HDL-C concentrations were 3.59 mmol/L, 0.81 mmol/L, 2.24 mmol/L and 0.82 mmol/L in the HLE+SLPC rats, respectively. The administration of SLPC significantly altered the abnormal biochemical parameter. These results demonstrated that the HLE model was successfully reproduced and the SLPC treatment could prevent or alleviate dyslipidemia.

Multivariate statistical analysis and lipid identification

The reproducibility of the UPLC-MS method was analyzed by six replicated determinations of the same plasma sample interspersed throughout the analysis. The extracted ion chromatographic peaks of ten ions (1.09_204.1235, 3.51_274.2744, 3.56_318.3003, 3.97_373.2733, 5.65_544.340, 5.62_520.3403, 6.09_496.3403, 6.49_522.3555, 7.28_546.3527, 7.56_524.3716) were selected for method validation. The RSD values of the retention time and peak area were below 0.54% and 2.9%, respectively. The results demonstrated good reproducibility.

Typical base peak intensity (BPI) chromatograms of the serum of diet-induced HLE rats were shown in Figure S1. The plasma metabolic profiling was acquired and analyzed by PLS-DA in positive ion mode. The PLS-DA scores plot was presented in Figure 1A. The corresponding loading plots (Figure 1B) showed candidate metabolites from CTL, HLE and HLE+SLPC groups. More than 3000 chromatographic peaks from three different groups were processed by MarkerLynx *XS* software to obtain better discrimination among CTL, HLE and HLE+SLPC groups. A clear separation between the CTL group and the HLE group was observed. The PLS-DA scores plot (Figure 1A) show the plasma lipid metabolic profile significantly changed in the diet-induced hyperlipidemic rats. Also, the data indicate the HLE+SLPC group located between the CTL group and the HLE group, and closer to the CTL group. The results reveal the general metabolic information was changed by the SLPC treatment.

Using the PLS-DA model from the 403 ions with high variable importance in the projection (VIP>1.5), a total of fifty ions were identified in this study and thirty-four lipid metabolites were selected. These lipid metabolites include seventeen glycerophospholipids, ten fatty acids or fatty acid esters, three sphingolipids, two glycerolipids

and one sterol lipid were identified based on criteria established in prior published studies [26,27,28] and are shown in Table 1. The lipids LPC(16:0), LPC(17:0), LPC(18:0), LPC(18:1), LPC(18:2), LPC(18:3), LPC(20:3), LPC(20:4), LPE(22:5), MG(16:1), palmitic acid, phytosphingosine, L-acetylcarnitine, cervonoyl ethanolamide, dodecanoic acid, 3-hydroxy-tetradecanoic acid and 12-O-20-carboxy-leukotriene B4 have been reported in the diet-induced hyperlipidemic rats [24]. Additional lipid metabolites were identified in the current study. Heatmap show the CTL, HLE and HLE+SLPC groups could be clearly separated based on thirty-four lipid metabolites (Figure 1C). These results were consistent with PLS-DA loading plots of the CTL, HLE and HLE+SLPC groups.

Selection lipid biomarkers of antihyperlipidemic effects of SLPC

To further select the potential biomarkers associated with HLE model and antihyperlipidemic effects of SLPC, univariate analyses including one-way ANOVA and nonparametric test Mann-Whitney U-test were performed in the selection of differentiating lipid metabolites to compare the HLE vs CTL group and SLPC vs HLE group. To account for multiple comparisons, *p* values were adjusted using the Hochberg and Benjamini FDR at $p<0.05$ to distinguish statistically significant lipid metabolites that differed between the two groups [29]. Twenty lipid metabolites were selected with one-way ANOVA, Mann-Whitney U-test and adjusted *p*-values less than 0.05.

To narrow the scope of the biomarker pool, Hierarchical cluster analysis was employed to reveal the potential relationships among the lipid metabolites. These lipid metabolites were clustered based on their Pearson correlation coefficients (Figure 2A). Five major clusters were observed. Two lysophosphatidylethanolamines, LPE(22:5) and LPE(22:4), are distributed in cluster 1. Phytosphingosine and MG(16:1) are distributed in cluster 2. All lysophosphatidylcholines with different carbon chain length including LPC(18:2), LPC(22:6), LPC(18:0), LPC(20:3), LPC(20:4) and LPC(20:1) are distributed in cluster 3. Three lipid metabolites including sphinganine, 12-O-20-carboxy-leukotriene B4 and PGP(18:1/18:2) are distributed in cluster 4. Fatty acids or fatty acid esters including 3-hydroxy-tetradecanoic acid, dodecanoic acid, palmitic acid, lucidenic acid, oleoylcarnitine, L-palmitoylcarnitine and linoleyl carnitine are distributed in cluster 5. The same types of lipid metabolites are distributed in the same cluster and have similar changing trends. These results demonstrate the abnormal lysophosphatidylcholine metabolism and fatty acid metabolism contribute to diet-induced hyperlipidemic rats. The SLPC treatment could alleviate abnormal changes associated with HLE. Heatmap indicate the CTL, HLE and HLE+SLPC groups could be completely separated and the HLE+SLPC group is located between the CTL and HLE groups (Figure 2B). The PCA and dendrogram analyses show the HLE+SLPC group is much closer to the CTL group compared with the HLE group (Figure 2C and D). The results show the lipid metabolites

significantly increase in the HLE group compared with the CTL group, while increased lipid metabolites are significantly reversed by the treatment with SLPC.

Two additional methods were used to specify select biomarkers and to clearly characterize the antihyperlipidemic effects of SLPC. First, the correlation coefficient analysis was applied to study the connections between identified lipid metabolites and their corresponding groups (Figure 3). Variables situated upper are positively correlated to corresponding group and those situated opposite are negatively correlated to the corresponding group. The lipid metabolites PGP(18:1/18:2) have a positive correlation with the CTL group. The other groups have a negative correlation with the CTL group, indicating normal metabolism of plasma lipid metabolites in the CTL rats. All of the lipid metabolites have a positive correlation with the HLE group, showing the overall metabolic profile of a high fat diet causing significant abnormal metabolism. The lipid metabolites LPE(22:5), 12-Oxo-20-CLB and LPE(22:4) have a positive correlation with the HLE+SLPC group. The other groups have a negative correlation with the HLE+SLPC group. Compared with the CTL group, the sixteen lipid metabolites LPC(18:2), LPC(18:0), LPC(20:4), palmitic acid, phytosphingosine, LPC(20:3), dodecanoic acid, sphinganine, 3-Hydroxy-tetradecanoic acid (3-HTA), L-palmitoylcarnitine, LPC(22:6), MG(16:1), oleoylcarnitine, lucidenic acid A, linoleyl carnitine and LPC(20:1) showed the same tendencies in the HLE+SLPC group (Figure 3). The lipid metabolites were reversed by treatment of SLPC on the diet-induced hyperlipidemia. These sixteen lipid metabolites may be considered as potential biomarkers for antihyperlipidemic effects of SLPC. To further investigate the antihyperlipidemic effects of SLPC of these specific biomarkers, PLS-DA-based ROC curves were performed. The area under the curve (AUC), 95% confidence interval (95%CI), sensitivities and specificities are shown in Figure 4. When the values of AUC, sensitivity and specificity were more than 0.85, 85% and 85%, respectively, the lipid metabolites were considered as potential biomarkers. Eleven lipid metabolites had an AUC of more than 0.85. Among these identified potential lipid metabolites, palmitic acid, phytosphingosine, dodecanoic acid, L-palmitoylcarnitine, oleoylcarnitine and linoleyl carnitine have a high sensitivity (>85%) and specificity (>85%) for predicting antihyperlipidemic effects of SLPC. Although four lysophosphatidylcholines, LPC(18:2), LPC(20:4), LPC(20:3) and LPC(22:6), have a high AUC value, their specificities were found to be low and thus they were not considered to be a suitable plasma biomarker for the prediction of an antihyperlipidemic effect of SLPC. These results indicate that fatty acyl lipid metabolites could be used as potential biomarkers in plasma for the prediction of an antihyperlipidemic effect of SLPC.

Biological pathway analysis with MetPA

The Ingenuity Pathway Analysis (IPA) is a web-based tool that combines an advanced pathway enrichment analysis with a pathway topology analysis to help investigators identify the most relevant metabolic pathway. MetPA uses a high-quality KEGG metabolic pathway as the backend knowledgebase. MetPA also implements a Google-map style interactive visualization system to provide the results in an intuitive manner. The biological pathway structure represented our knowledge about the complex interaction relationships from different molecules within a cell or a living organism. Well-known changes in more important positions of a network will trigger a more severe impact on the pathway than changes occurring in a marginal or relatively isolated position. Biological pathway analysis with MetPA shows the lipid metabolites identified together are important for the diet-induced HLE and the antihyperlipidemic effect of SLPC. They are responsible for fatty acid metabolism, fatty acid biosynthesis, sphingolipid metabolism, fatty acid elongation in mitochondria and unsaturated fatty acids biosynthesis (Figure 5 and Table 2).

Using the IPA analysis, we characterized six lipid metabolites, palmitic acid, phytosphingosine, dodecanoic acid, L-palmitoylcarnitine, oleoylcarnitine and linoleyl carnitine, to plot the significantly change of biochemical metabolism induced by the high fat diet and antihyperlipidemic effect of SLPC. Figure 6 as an example shows the detailed results from the biological pathway analysis of fatty acids metabolism. The others were shown in Figure S2, S3, S4 and S5. These results suggest that significant perturbations of these lipid metabolites occur in the above-mentioned metabolic pathways.

Biochemical interpretation

The LIPID MAPS consortium defined lipids as hydrophobic or amphipathic small molecules that originate entirely or in part by carbocation based condensations of isoprene group or by carbanion based condensations of ketoacyl group [30]. Based on this definition, lipids can be divided into eight categories: fatty acyls, glycerolipids, sphingolipids, glycerophospholipids, saccharolipids, sterol lipids, prenol lipids and polyketides [30]. Figure 7 represents the change of the six lipid metabolites among the CTL, HLE and HLE+SLPC groups. The results indicate six lipid metabolites were considered as the biomarkers of antihyperlipidemic effects of SLPC, which are associated with the biochemical mechanism of SLPC. In this study, we observed a significant increase of five fatty acyl metabolites, palmitic acid, dodecanoic acid, L-palmitoylcarnitine, oleoylcarnitine and linoleyl carnitine, as well as, a significant increase in sphingolipid phytosphingosine in the high fat diet-induced

hyperlipidemic rats. However, the six lipid metabolites significantly decreased with treatment of SLPC.

Fatty acid metabolism- Fatty acids are the simplest and one of the most important lipid molecular classes. They are a basic component of all lipid molecules. Fatty acids are present in the form of saturated fatty acids (SFA) and unsaturated fatty acids in the human. The perturbation of fatty acid metabolism has an important effect on the initiation and progression of dyslipidemia and coronary heart disease [31]. Recent studies show SFA metabolism perturbed in HLE [32,33]. Hyperlipidemic rats were fed a high-fat diet, which contain a high SFA content of lard and egg yolk powder. Excessive intake of SFA caused a significant increase in TC, TG and LDL-C, and this is one of important reasons for atherosclerosis and an increased risk of suffering from coronary heart disease. Compared with the HLE rats, significantly increased plasma SFA were reversed by treatment with SLPC and the reason was not clear completely.

The previous study demonstrated an increase in blood and liver SFA promoted β-oxidation, leading to greater amounts of acetyl-CoA. Part of the acetyl CoA contributed to the tricarboxylic acid cycle to generate energy, and the rest participated in ketone bodies and cholesterol, causing increased blood ketone bodies and cholesterol [31]. It has been reported the activity of liver HMG-CoA reductase increased after excessive intake of SFA that increased cholesterol synthesis [34]. Another study reported an increase in fatty acids in plasma than in the liver tissue [35]. This may be attributed to fatty acids synthesis in the liver promptly converting to triglyceride by hepatic diglycerol acyltranferase and exported in the circulation by very low density lipoprotein. Plasma lipidomics show an increase level of palmitic acid, dodecanoic acid, L-palmitoylcarnitine, oleoylcarnitine and linoleyl carnitine in the diet-induced hyperlipidemic rats. Our results are consistent with a previous study in rats, in which increased absorption of lipids resulted in increased plasma fatty acids [36]. These results indicate SLPC regulated abnormal SFA metabolism on the diet-induced hyperlipidemia by the inhibition of HMG-CoA reductase activity.

Our study identified three fatty acyl carnitines as the main biomarkers in the diet-induced hyperlipidemia and antihyperlipidemic effect of SLPC. The Carnitine cycle is the first step for the biochemical reaction of fatty acid oxidation, in which the fatty acyl CoA enters the mitochondria as fatty acyl carnitine by the carnitine transport [37]. Under dyslipidemia condition, the abnormal glycolysis accelerates the reaction of fatty acid oxidation to supply the required energy, in which fatty acyl carnitine is metabolized to promote the long-chain fatty acid in the mitochondria. It has been reported that excessive intake of cholesterol upregulates the gene expression of the fatty acid oxidation in ApoE*3 Leiden transgenic mice, which provide the indirect evidence for their promotion

of fatty acids oxidation [38]. Furthermore, study reported the inhibition of fatty acid metabolism caused an accumulation of toxic intermediates such as long-chain acylcarnitine derivatives [39].

Sphingolipid metabolism-Phytosphingosine plays an important role in the sphingolipid biosynthesis and metabolism. Phytosphingosine is a highly bioactive metabolite and participates in cell proliferation, differentiation and apoptosis processes. Phytosphingosine induces apoptosis in human non-small cell lung cancer cells and T-cell lymphoma cells, and induces caspase-independent cytochrome c release from mitochondria. Our study show phytosphingosine in plasma significantly increase in the diet-induced hyperlipidaemic rats and significantly decrease by treatment with SLPC. An increase in phytosphingosine in plasma was consistent with reported results from an atherosclerosis model [40]. Sphingomyelinase hydrolyse sphingomyelin causes ceramide release and phytosphingosine accumulation [41]. Significant increase of sphingolipids may reduce reverse cholesterol transport and thereby increase risk of hyperlipidemia-related diseases [42,43]. A significant increase in phytosphingosine in the diet-induced hyperlipidemic rats demonstrates the expression of sphingomyelinase was upregulated and enhanced sphingolipid metabolism with the consumption of a high fat diet. Based on our current study, compared with the diet-induced hyperlipidemic rats, phytosphingosine decreased by treatment with SLPC, which may show dyslipidemia progress is involved in phytosphingosine metabolism.

Conclusion

The antihyperlipidemic effect of SPLC was evaluated in the high fat diet-induced hyperlipidemic rats using a UPLC-QTOF/HDMS-based lipidomic approach. Pathological and pharmacological biomarkers were used to construct the metabolic pathways underlying the pharmacological bioactivity of the antihyperlipidemia of SPLC. Twenty-five lipid metabolites significantly varied between the HLE group and the CTL group. These were characterized as potential pathological biomarkers associated with HLE. Twenty-seven lipid metabolites varied between the HLE group and the HLE+SLPC group and were characterized as potential biomarkers for the antihyperlipidemic effect of SPLC. Six lipid metabolites including five fatty acyl lipid metabolites palmitic acid, dodecanoic acid, L-palmitoylcarnitine, oleoylcarnitine and linoleyl carnitine and one sphingolipid phytosphingosine were considered as biomarkers of diet-induced hyperlipidemia and antihyperlipidemic effect of SLPC. These findings suggest a high fat diet led to the perturbation of fatty acid metabolism and sphingolipid metabolism, which may be the pharmacological basis of antihyperlipidemic effect of SLPC.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

Acknowledgments

This study was supported by the Program for the New Century Excellent Talents in University (No. NCET-13-0954) from Ministry of Education of China, the National Natural Science Foundation of China (Nos. J1210063, 81202909, 81274025, 81001622) and the project "As a Major New Drug to Create a Major National Science and Technology Special" (No. 2014ZX09304307-002).

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Table 1. Identified plasma lipids, fold changes (FC) and *p*-values among CTL, HLE and HLE+SLPC groups

No.	metabolites	VIP[a]	HLE vs CTL				SLPC vs HLE				SLPC vs CTL			
			FC[b]	*p*-value[c]	*p*-value[d]	FDR[e]	FC[a]	*p*-value[b]	*p*-value[c]	FDR[d]	FC[a]	*p*-value[b]	*p*-value[c]	FDR[d]
1	LPC(16:0)	8.7	-0.08	3.17E-01	2.70E-01	3.47E-01	0.34	5.50E-04	1.63E-03	1.56E-03	0.25	2.56E-03	8.65E-03	7.91E-03
2	LPC(18:2)	8.4	0.38	2.26E-05	1.12E-03	4.51E-05	-0.34	1.08E-03	3.25E-03	2.44E-03	0.04	5.78E-01	6.74E-01	5.78E-01
3	LPC(18:0)	7.7	0.58	2.68E-08	7.71E-04	4.55E-07	-0.38	2.59E-07	7.64E-04	8.81E-06	0.21	4.34E-03	8.60E-03	9.85E-03
4	LPC(18:1)	6.7	0.28	3.30E-02	4.58E-02	4.32E-02	-0.43	3.65E-04	1.62E-03	1.38E-03	-0.15	2.80E-01	1.88E-01	2.88E-01
5	LPC(20:4)	6.4	0.66	7.67E-07	7.78E-04	3.73E-06	-0.27	8.23E-04	3.25E-03	2.00E-03	0.38	3.22E-03	6.28E-03	9.12E-03
6	MG(18:2)	6.1	0.34	3.80E-01	3.98E-01	4.04E-01	1.87	1.42E-04	7.23E-04	8.03E-04	2.21	1.19E-02	2.34E-02	1.50E-02
7	Palmitic acid	5.8	0.71	6.30E-06	7.71E-04	1.53E-05	-0.29	5.22E-04	7.71E-04	1.61E-03	0.42	7.43E-03	1.57E-02	1.20E-02
8	Phytosphingosine	5.1	0.86	2.70E-07	7.78E-04	2.30E-06	-0.39	1.69E-06	7.78E-04	2.87E-05	0.46	8.03E-04	1.93E-03	3.41E-03
9	LPC(20:3)	3.6	0.95	3.01E-07	7.43E-04	2.05E-06	-0.48	7.09E-04	3.77E-03	1.86E-03	0.47	3.22E-03	4.40E-03	8.43E-03
10	L-Acetylcarnitine	3.3	0.11	7.47E-01	4.27E-01	7.47E-01	0.37	4.46E-02	5.15E-02	5.42E-02	0.48	5.23E-02	3.50E-02	6.13E-02
11	Cervonoyl ethanolamide	3.2	0.63	5.54E-01	3.09E-01	5.70E-01	1.21	1.35E-02	2.00E-02	2.00E-02	1.84	7.41E-03	1.50E-02	1.26E-02
12	Dodecanoic acid	3.2	0.94	6.85E-06	7.57E-04	1.55E-05	-0.40	4.25E-04	2.61E-03	1.45E-03	0.54	3.97E-03	1.75E-02	9.65E-03
13	TG(18:0/16:1/20:0)	2.8	-0.56	8.69E-04	3.01E-03	1.28E-03	-0.26	6.58E-02	2.26E-01	7.71E-02	-0.81	8.81E-09	6.29E-04	3.00E-07
14	Sphinganine	2.7	0.54	3.88E-04	5.28E-03	6.00E-04	-0.16	2.11E-02	2.54E-02	2.99E-02	0.37	7.89E-03	1.13E-02	1.22E-02
15	LPC(18:3)	2.6	0.39	8.43E-05	9.85E-04	1.51E-04	-0.09	1.20E-01	6.17E-02	1.35E-01	0.30	6.22E-03	9.75E-03	1.18E-02
16	3-Hydroxy-tetradecanoic acid	2.5	0.95	4.56E-06	7.37E-04	1.19E-05	-0.44	2.92E-04	1.54E-03	1.24E-03	0.50	8.57E-03	1.51E-02	1.21E-02
17	L-Palmitoylcarnitine	2.4	2.46	1.06E-05	6.78E-04	2.26E-05	-0.54	1.24E-02	1.72E-02	1.91E-02	1.92	2.13E-06	6.65E-04	2.41E-05
18	LPC(17:0)	2.4	-0.22	2.00E-01	3.19E-01	2.27E-01	1.23	1.75E-04	5.43E-04	8.48E-04	1.01	2.44E-03	6.06E-03	8.28E-03
19	LPC(22:6)	2.4	0.63	5.31E-05	7.57E-04	1.00E-04	-0.28	1.58E-03	3.16E-03	3.36E-03	0.35	5.50E-03	1.53E-02	1.10E-02
20	LPE(22:5)	2.3	0.38	2.73E-02	3.89E-02	3.72E-02	-0.71	4.64E-06	8.06E-04	5.26E-05	-0.33	7.39E-02	9.46E-02	8.11E-02
21	LPE(20:4)	2.3	-0.43	1.84E-03	3.65E-03	2.89E-03	0.06	3.51E-01	6.95E-01	7.22E-01	-0.47	7.56E-02	6.59E-02	7.89E-02
22	Crustecdysone	2.3	0.07	1.83E-01	1.84E-01	2.22E-01	0.12	2.59E-02	2.61E-02	3.52E-02	0.19	7.21E-03	1.34E-02	1.29E-02
23	LPC(16:1)	2.2	0.64	2.89E-06	7.43E-04	9.82E-06	-0.05	5.69E-01	5.24E-01	5.87E-01	0.58	7.61E-05	1.06E-03	3.69E-04
24	MG(16:1)	2.2	0.61	1.32E-04	1.55E-03	2.24E-04	-0.24	1.73E-03	5.94E-03	3.45E-03	0.37	9.55E-03	1.94E-02	1.30E-02
25	LPE(18:2)	2.1	0.09	1.50E-01	1.68E-01	1.89E-01	0.11	3.09E-02	3.95E-02	4.04E-02	0.20	8.02E-03	9.97E-03	1.19E-02
26	12-O-20-carboxy-leukotriene B4	2.0	1.62	1.30E-06	6.78E-04	5.50E-06	-0.90	1.10E-04	1.61E-03	7.50E-04	0.71	9.78E-03	4.03E-03	1.28E-02
27	Oleoylcarnitine	2.0	2.91	3.62E-06	7.30E-04	1.12E-05	-0.83	2.25E-03	3.50E-03	4.24E-03	2.08	2.49E-05	7.04E-04	1.41E-04
28	PGP(18:1/18:2)	2.0	2.96	1.61E-08	4.85E-04	5.48E-07	-0.55	5.63E-03	8.24E-03	1.01E-02	2.42	2.04E-05	7.69E-04	1.39E-04
29	LPE(22:4)	1.9	0.90	2.54E-07	7.04E-04	2.88E-06	-0.32	6.53E-03	1.38E-02	1.11E-02	0.58	1.09E-03	2.94E-03	4.13E-03
30	LPC(20:5)	1.9	1.11	4.55E-06	6.72E-04	1.29E-05	-0.38	2.02E-01	7.86E-02	2.15E-01	0.73	2.09E-02	2.60E-02	2.54E-02
31	Dihydroceramide	1.8	0.45	3.48E-04	1.87E-03	5.64E-04	-0.11	1.58E-01	1.66E-01	1.73E-01	0.34	5.25E-03	1.30E-02	1.12E-02
32	Lucidenic acid A	1.8	2.47	5.86E-07	5.32E-04	3.32E-06	-0.19	3.23E-02	3.65E-02	4.07E-02	2.27	4.22E-06	4.32E-04	3.58E-05
33	Linoleyl carnitine	1.8	2.92	2.09E-06	6.84E-04	7.90E-06	-0.66	7.44E-03	1.44E-02	1.20E-02	2.25	9.55E-08	4.80E-04	1.62E-06
34	LPC(20:1)	1.7	0.38	1.21E-03	4.11E-03	1.72E-03	-0.59	4.82E-05	8.60E-04	4.10E-04	-0.20	6.72E-02	7.68E-02	7.61E-02

[a] VIP value was obtained from PLS-DA model; [b] The fold change (FC) was calculated based on binary logarithm for HLE vs CTL, SLPC vs HLE or SLPC vs CTL. FC with a value greater than zero indicates a higher intensity of the plasma metabolite between HLE vs CTL, between SLPC vs HLE or between SLPC vs CTL, while a FC value less than zero indicates a lower intensity of the plasma metabolite between HLE vs CTL, between SLPC vs HLE or between SLPC vs CTL; [c] *p*-values are calculated from a one-way ANOVA; [d] *p*-values are calculated from nonparametric test Mann-Whitney U-test; [e] The false discovery rate (FDR) value was obtained from the adjusted *p* value of FDR correction using Benjamini Hochberg method.

Table 2 Ingenuity pathway analysis (A) and sphingolipid metabolism (B) with MetPA from lipid metabolites

Pathway Name	Total metabolites	Hits	*p*	-log(*p*)	Holm *p*	FDR	Impact	Details
Fatty acid metabolism	39	2	0.0043	5.45	0.35	0.21	0.0	Figure 6
Fatty acid biosynthesis	43	2	0.0052	5.26	0.42	0.21	0.0	Figure S2
Sphingolipid metabolism	21	1	0.0580	2.84	1.00	1.00	0.0	Figure S3
Fatty acid elongation in mitochondria	27	1	0.0741	2.60	1.00	1.00	0.0	Figure S4
Unsaturated fatty acids biosynthesis	42	1	0.1135	2.17	1.00	1.00	0.0	Figure S5

Total is the total number of lipid metabolites in the pathway; the Hits is the actually matched number from the user lpid metabolites; the Raw *p* is the original *p* calculated from the enrichment analysis; the Holm *p* is the *p* value adjusted by Holm–Bonferroni method; the impact is the pathway impact value calculated from pathway topology analysis.

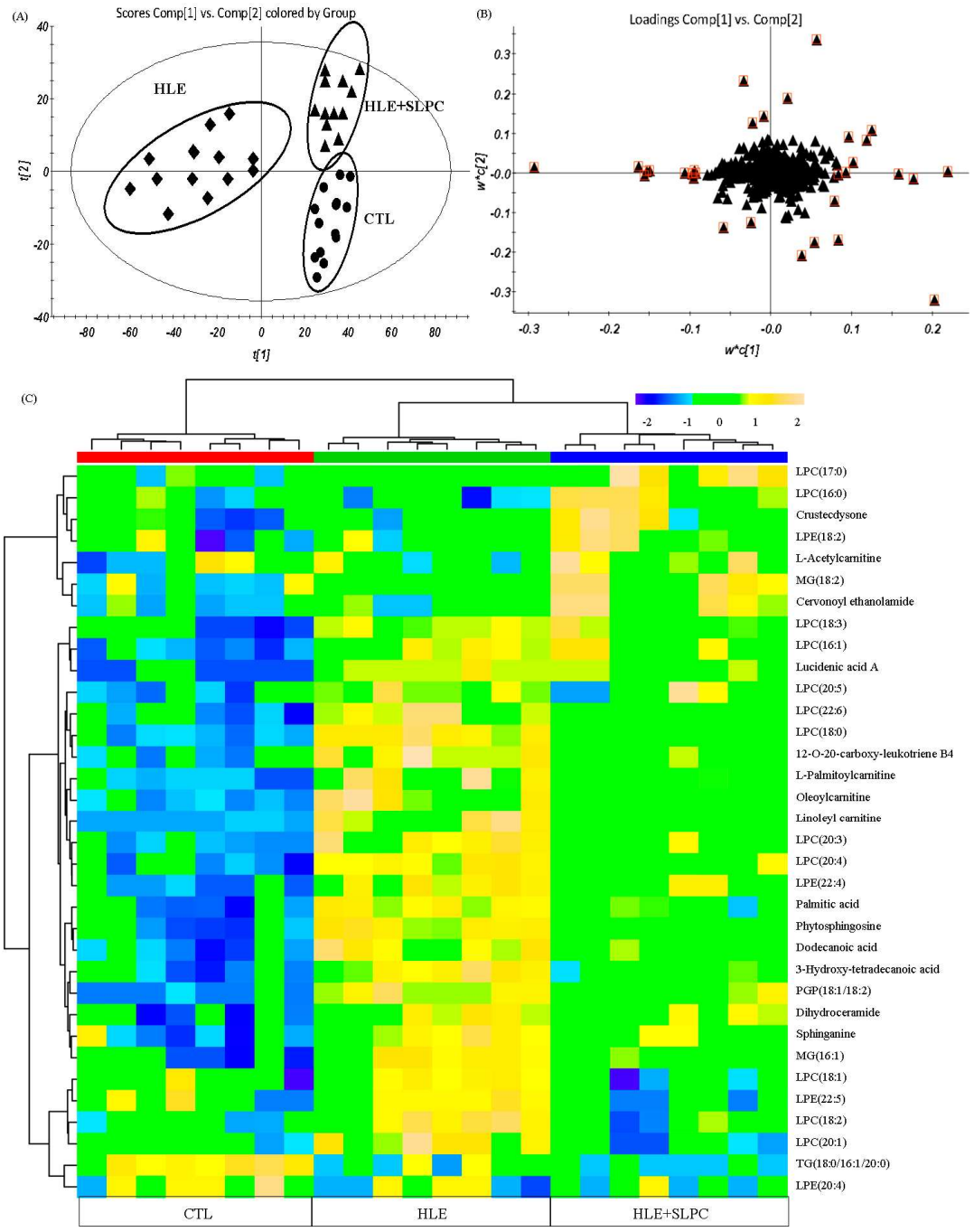

Figure 1. Multivariate statistical analysis of the CTL, HLE and HLE+SLPC groups. PLS-DA scores plot (A) loading plot (B) of plasma from CTL, HLE and HLE+SLPC groups. The variables marked are the metabolites selected as potential biomarkers. (C) Hierarchical clustering heat map of the thirty-four differential lipids, with the degree of change in the CTL, HLE and HLE+SLPC groups marked with colors indicating up-regulation (pink) and down-regulation (blue). Identified lipids were represented in the horizontal axis, and individual samples in the vertical axis. *t[1]*, component 1; *t[2]*, component 2; *w*c[1]*, component 1; *w*c[2]*, component 2; comp[1], component 1; comp[2], component 2.

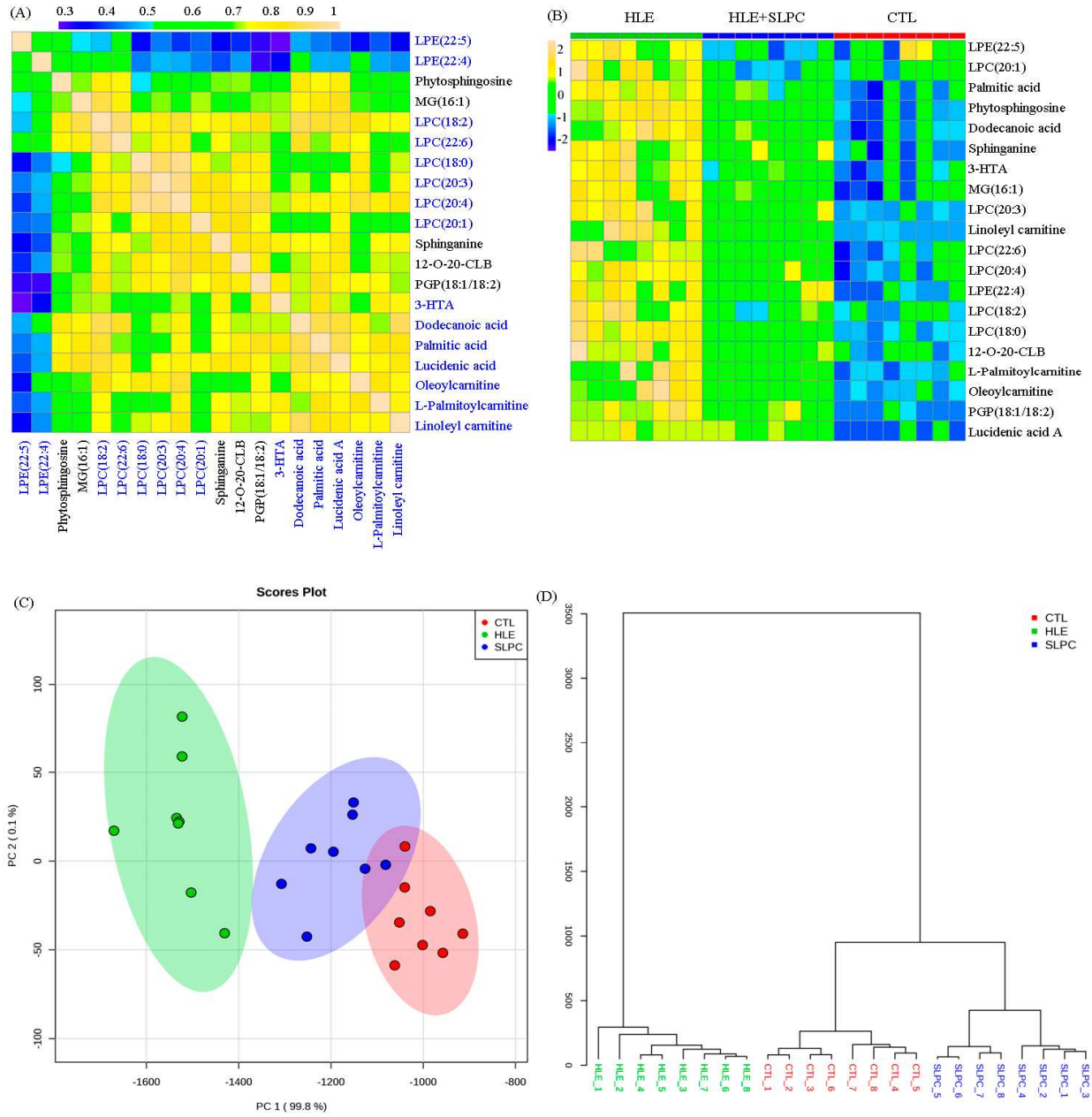

Figure 2. Hierarchical cluster of twenty identified lipid metabolites. (A) Correlation analysis of the differential lipid metabolites in the CTL, HLE and HLE+SLPC groups. (B) Heat map cluster for identified plasma lipid metabolites in the CTL, HLE and HLE+SLPC groups. The color of each section is proportional to the significant change in lipid metabolites (pink, up-regulation; blue, down-regulation). 12-O-20-CLB: 12-O-20-carboxy-leukotriene B4; 3-HTA: 3-hydroxy-tetradecanoic acid. (C) PCA of twenty differential lipid metabolites. The HLE and control could be separated completely based on these identified lipid metabolites. (D) Dendrograms of hierarchical clustering of CTL, HLE and HLE+SLPC groups using significant metabolites by one-way ANOVA.

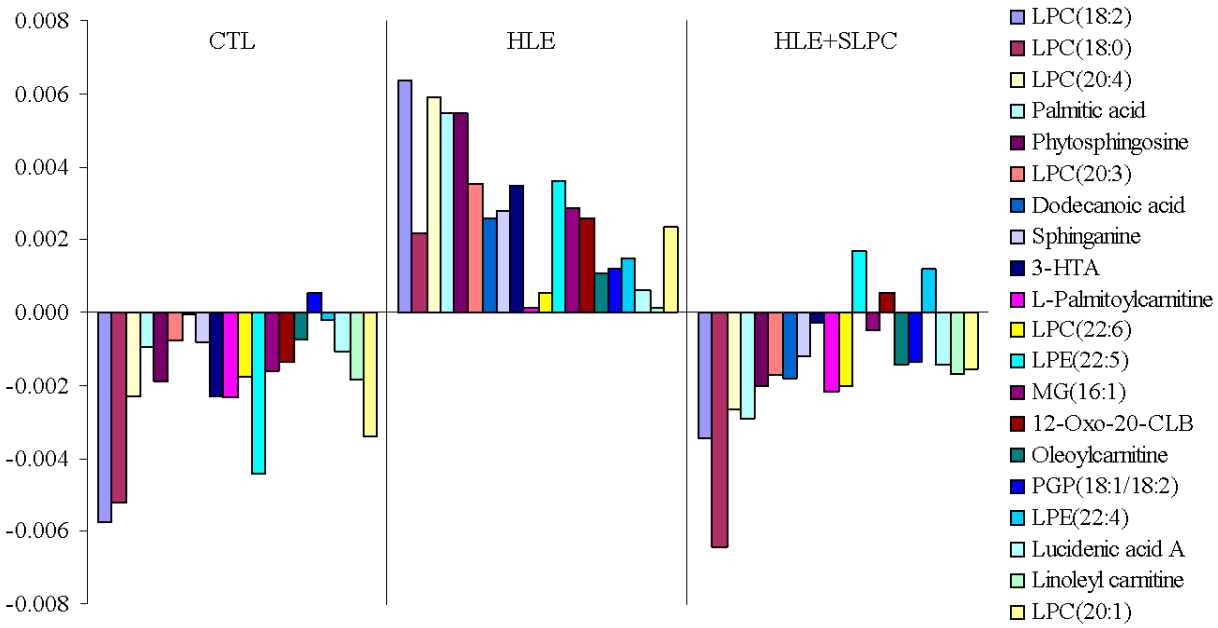

Figure 3. Correlation coefficient analysis among CTL, HLE and HLE+SLPC groups with corresponding lipid metabolites in the different groups. Variables are presented in CTL, HLE and HLE+SLPC groups. Values of correlations are shown in the vertical axis (upper for positive correlations and under for negative correlations) and corresponding lipid metabolites represented to the right of the bars.

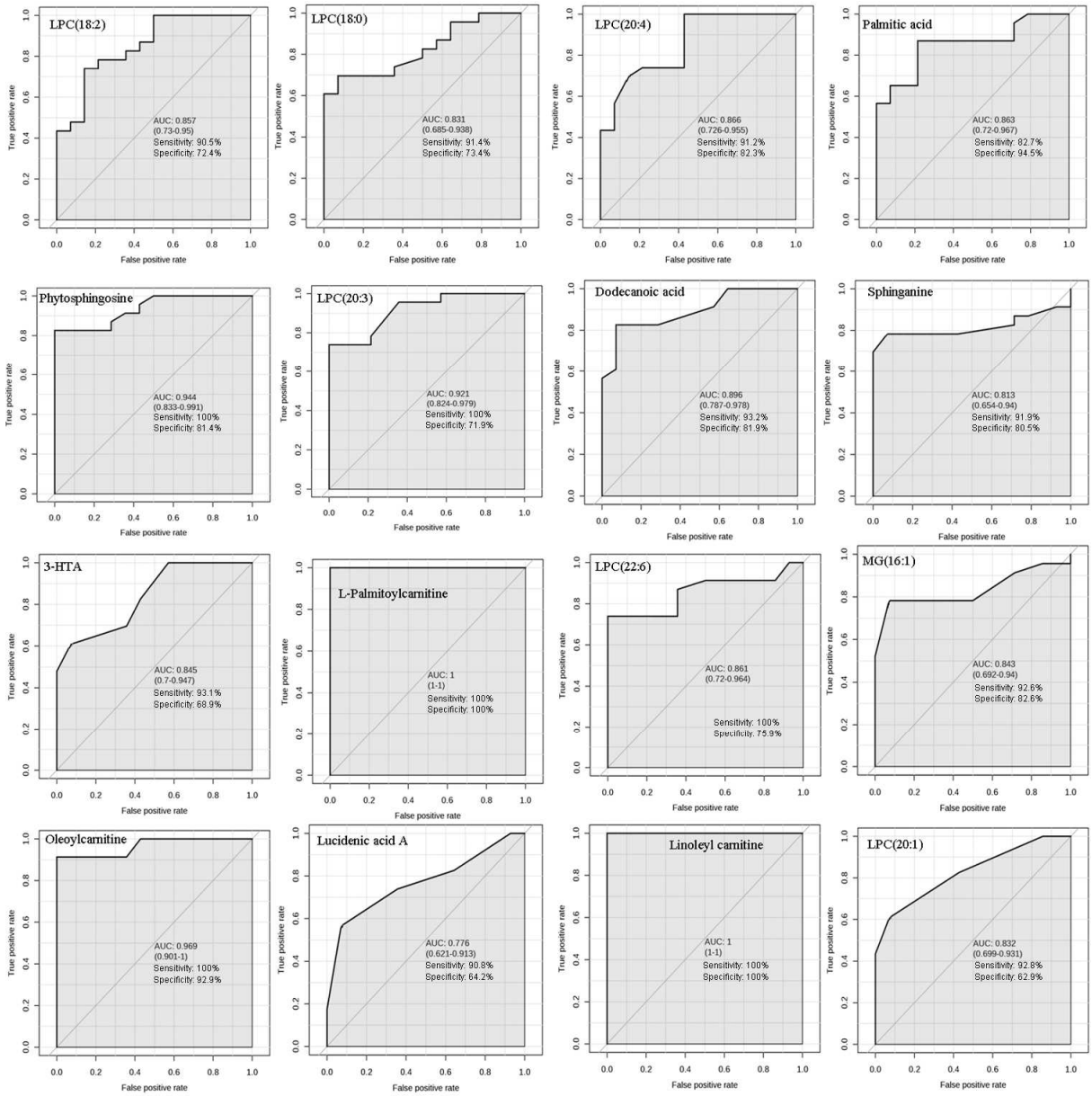

Figure 4. PLS-DA-based receive operating characteristic curves of the identified lipid metabolites for evaluation of antihyperlipidemic effects of SLPC. The associated area under the curve (AUC), 95% confidence interval (95%CI), sensitivities and specificities were indicated.

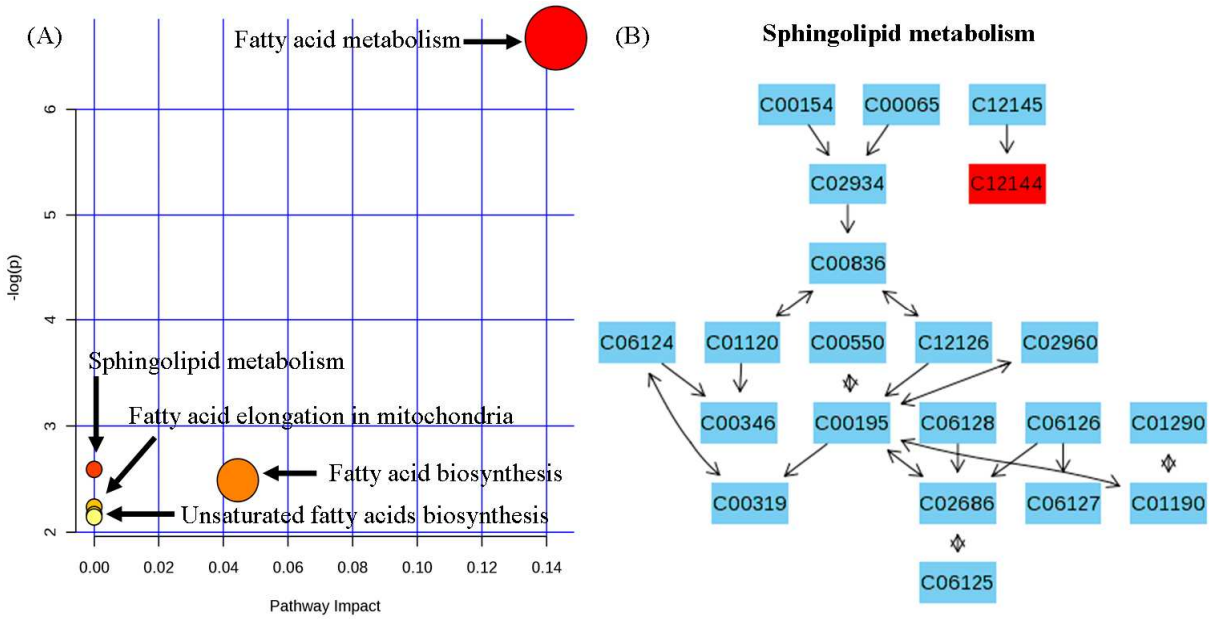

Figure 5. Ingenuity pathway analysis with MetPA. Summary of ingenuity pathway analysis with MetPA including fatty acid metabolism, fatty acid biosynthesis, sphingolipid metabolism, fatty acid elongation in mitochondria and unsaturated fatty acids biosynthesis with MetPA from significantly lipid metabolites (A) and sphingolipid metabolism (B). The size and color of each circle was based on pathway impact value and *p*-value, respectively.

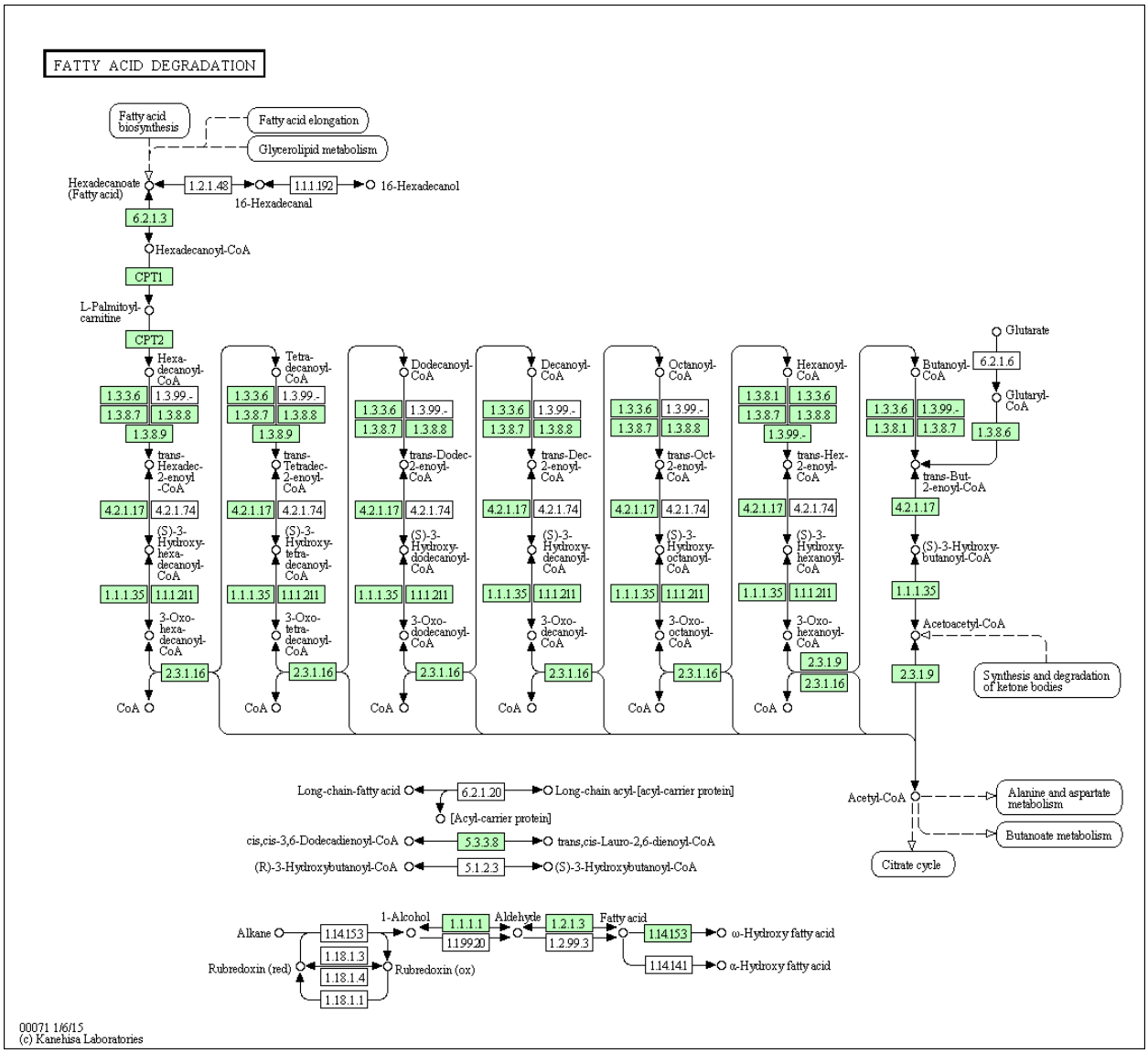

Figure 6. Overview of fatty acid metabolism with MetPA in rats. The reference map by KEGG (http://www.genome.jp/kegg/). The green boxes represent enzymatic activities with putative cases of analogy in rats.

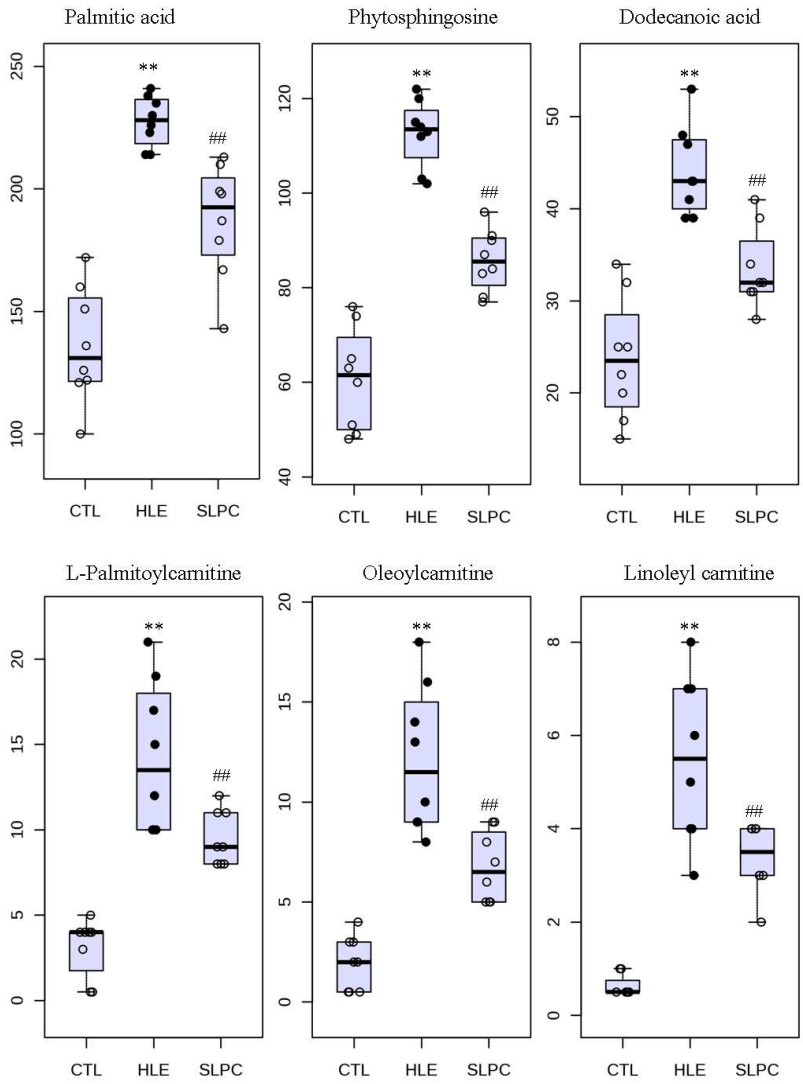

Figure 7. Combined box-and-whisker and dot plot of normalized intensity of six lipid metabolites (palmitic acid, phytosphingosine, dodecanoic acid, L-palmitoylcarnitine, oleoylcarnitine and linoleyl carnitine) in the CTL, HLE and HLE+SLPC groups. The statistical significance between the two groups are marked. **P<0.01 significant difference compared with CTL group; $^{\#\#}$P<0.01 significant difference compared with HLE group.

Graphical Abstract